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## 1. General procedures

Cell lines A20 (ATCC, TIB-208), HEK293 (ATCC, CRL-1573) and HeLa (ATCC, CCL-2) were obtained from American Type Culture Collection and cultured under recommended conditions. For A20 cells were kept at density below  $3 \times 10^5$ /ml using high quality FBS.

All animal procedures were performed according to US National Institutes of Health guidelines and approved by the Committee on Animal Care at MIT. 6- to 8- week old female Balb/c mice (Charles River) were maintained on normal diet and housed 5/cage. Mice for purpose of fluorescent imaging were maintained on Casein Diet 10 IF (LabDiet) at least one week before imaging.

DM1 was purchased from Carbosynth Ltd.; all reagents were obtained from commercial sources and used as purchase. Organic solvents were purchased anhydrous and used without further purification. Unless otherwise noted, all reactions were carried out at room temperature. LC-MS was recorded on a Waters Xevo system equipped with UPLC-C8 columns. MALDI-TOF was recorded on a Bruker Microflex system. The matrix used was 2,5-dihydroxy-benzoic acid (DHB).

## 2. Peptide Synthesis

According to the general method of peptide synthesis, sequences GGGC and GGGDTDTC with free N-terminals were synthesized using Rink amide resin (470 mg, 0.25 mmol, loading: 0.53 mmol/g). Commercially available Fmoc-G<sub>3</sub>-OH (Chem-Impex) was used to accelerate the synthesis.

1. Coupling condition: Fmoc-AA-OH (0.75 mmol) HATU (285 mg, 0.75 mmol), *N,N*-diisopropylethylamine (DIPEA) (263  $\mu$ L, 1.5 mmol), DMF (5 ml). After premixing for 1 min, the pre-activated solution was added and agitated for 2 h.

2. Deprotection condition: 20% piperidine in DMF (10 ml), 20 min.

3. Cleavage condition: TFA/TIPS/EDT/H<sub>2</sub>O (90/5/2.5/2.5), 2 h.

4. Purification and characterization: Cleavage solution (<10 ml) was precipitated in prechilled Ether (30 ml), centrifuged and the supernatant was removed. Peptides were directly dissolved in H<sub>2</sub>O followed by the purification by RP-HPLC on a semipreparative column (C18 column, Gemini, 5  $\mu$ m, 10  $\times$  250 mm; Phenomenex) at a flow rate of 3.0 mL/min: solvent A, 0.1% TFA in H<sub>2</sub>O; solvent B, 0.1% TFA in CH<sub>3</sub>CN. Gradient: 5%  $\rightarrow$  90% in 15 min. Fractions containing pure product were collected and lyophilized. LC-MS calculated for GGGC: C<sub>9</sub>H<sub>17</sub>N<sub>5</sub>O<sub>4</sub>S [M+H]<sup>+</sup> was 292.1079, found 292.1093. GGGDTDTC: C<sub>25</sub>H<sub>40</sub>N<sub>8</sub>O<sub>15</sub>S [M+H]<sup>+</sup> was 725.2412, found 725.2443.



## 2.1 Synthesis of GGGDTDTC-BMOE

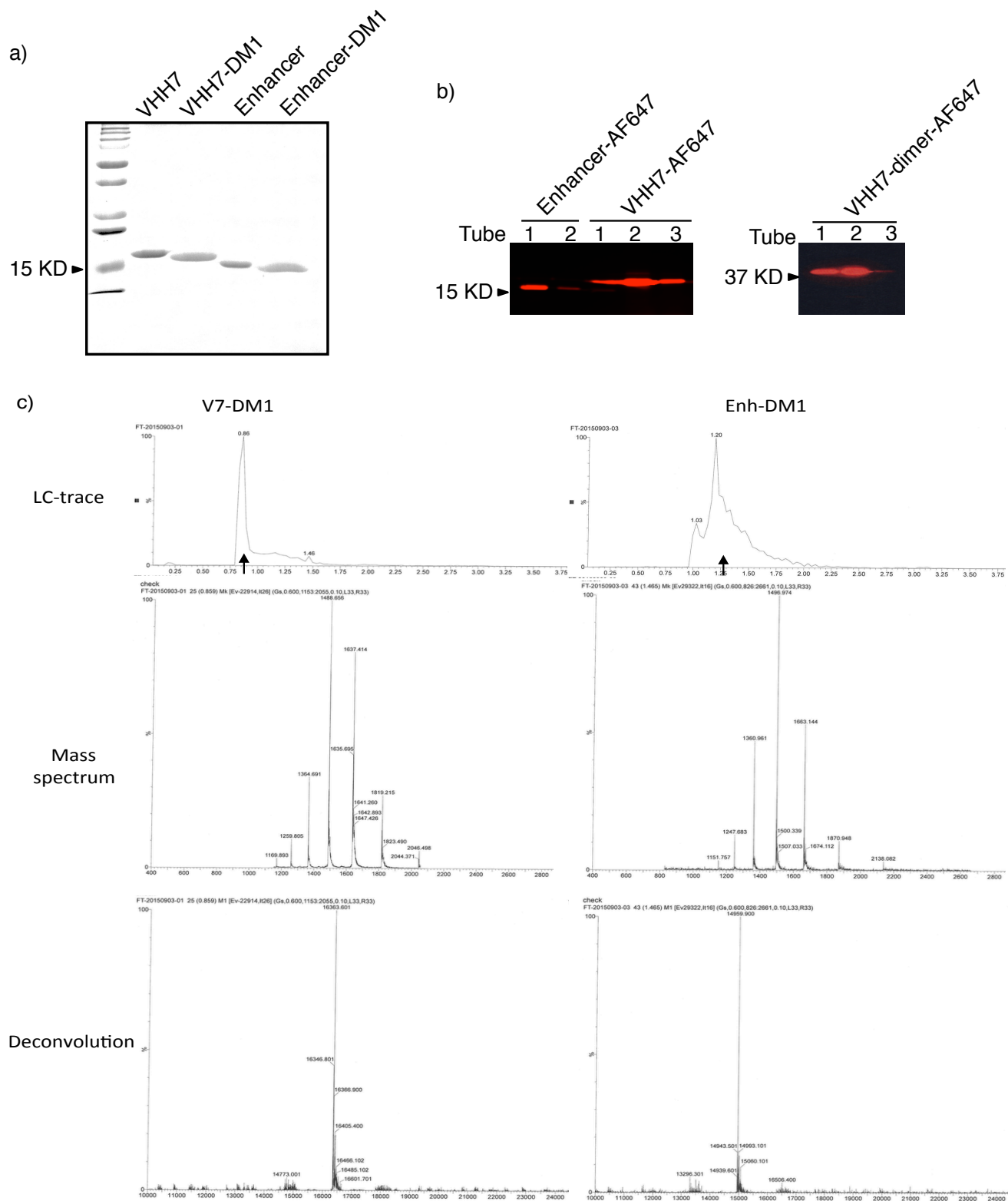
Peptide GGGDTDTC (10 mg, 0.014 mmol) and bismaleimidoethane (BMOE) (15 mg, 0.07 mmol) were dissolved in DMF (0.5 ml) separately. Once BMOE was fully dissolved, peptide solution was added slowly followed by shaking at r.t. for 2 h. The completion of the reaction was confirmed by LC-MS as the disappearance of original peptide. Excess BMOE was precipitated by the addition of H<sub>2</sub>O (1 ml) and incubation at -20 °C for 20 min. The solution was centrifuged and the supernatant was purified by RP-HPLC on a semipreparative column (C18 column, Gemini, 5 µm, 10 × 250 mm; Phenomenex) at a flow rate of 3.0 mL/min: solvent A, 0.1% TFA in H<sub>2</sub>O; solvent B, 0.1% TFA in CH<sub>3</sub>CN. Gradient: 5% → 90% in 15 min. Fractions containing pure product were collected and lyophilized. LC-MS calculated for: C<sub>33</sub>H<sub>45</sub>N<sub>11</sub>O<sub>18</sub>S [M+H]<sup>+</sup> was 916.2743, found 916.2789.

## 2.2 Synthesis of GGGDTDTC-BMOE-DM1

GGGDTDTC-BMOE (1 mg, 1.1 µM) was dissolved in PBS<sup>-</sup> (200 µl). DM1 (2.4 mg, 3.2 µM) was dissolved in DMF (300 µl) then added to peptide solution. After shaking at 18 °C for 16 h, the reaction mixture was directly purified by RP-HPLC on a semi-preparative column (C18 column, Gemini, 5 µm, 10 × 250 mm; Phenomenex) at a flow rate of 3.0 mL/min: solvent A, 0.1% TFA in H<sub>2</sub>O; solvent B, 0.1% TFA in CH<sub>3</sub>CN. Gradient: 5% → 90% in 15 min. Fractions containing pure product were collected and lyophilized. LC-MS calculated for: C<sub>70</sub>H<sub>97</sub>ClN<sub>14</sub>O<sub>28</sub>S<sub>2</sub> [M+H]<sup>+</sup> was 1681.5805, found 1681.5786.

## 3. Site-specific modifications of VHHs by sortagging

The pentamutant sortase A, with an improved K<sub>cat</sub>, was used (addgene: 51140). Reaction mixtures (1 mL) contained Tris·HCl (50 mM, pH 7.5), CaCl<sub>2</sub> (10 mM), NaCl (150 mM), triglycine-containing probe (500 µM), LPETG-containing VHHs (100 µM), and sortase (2.5 µM). After incubation at 12 °C with agitation for 2~4 h, Ni-NTA beads were added to the reaction mixture with gentle shaking for 30 min at 4 °C, followed by centrifugation to remove sortase and any remaining unreacted His-tagged substrate. The final product was purified by size-exclusion chromatography in PBS or Tris·HCl (50 mM, pH 7.5). The labeled protein was stored at -80 °C with 10% (vol/vol) glycerol.

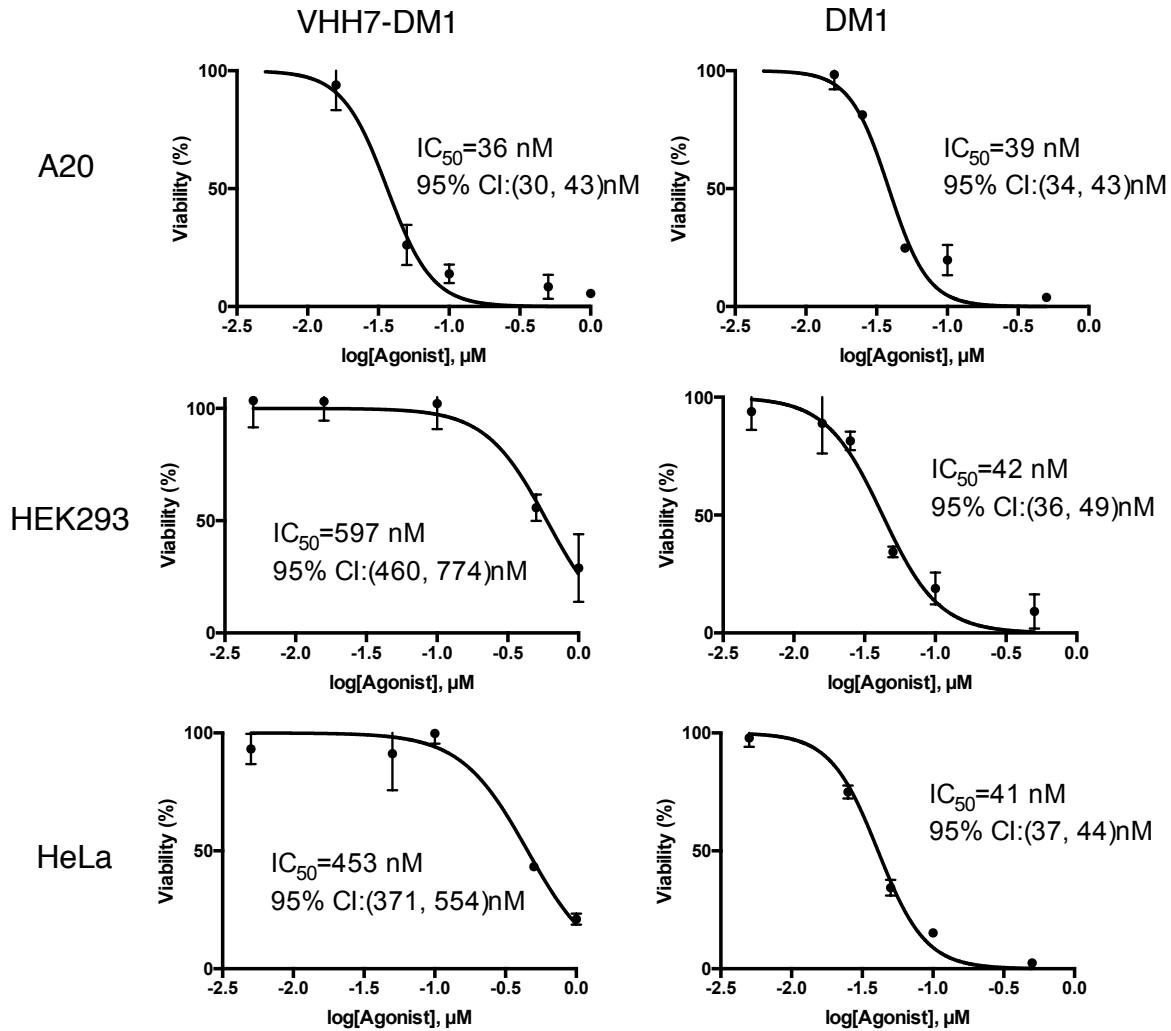


#### **4. Determine half maximum binding affinity by FACS**

A20 cells were harvested from suspension followed by centrifugation and resuspension in FACS buffer (2% FCS, 2 mM EDTA in PBS<sup>-</sup>) to density  $1 \times 10^6$ /ml and transferred to FACS tubes (1 ml/tube). VHH7-AF647 conjugate (0.38 mg/ml) was added directly to each tube to final concentrations of 0.3 nM, 1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 300 nM, 1  $\mu$ M. After incubation on ice for 20 min, the cells were pelleted by centrifugation and washed (3  $\times$  FACS buffer). The samples were kept in FACS buffer on ice until analysis. The binding of VHH7 to A20 was analyzed on BD Cantoll with 50,000 events/sample. Data was analyzed using FlowJo based on mean fluorescence intensity.

#### **5. *In vitro* cell cytotoxicity assay of VHH7-DM1**

The *in vivo* cytotoxicity of VHH7-DM1 conjugates and their selectivity on MHC-II negative cell lines were tested using MTT cell proliferation assay (Life Technologies). Cells were seeded in flat bottom 96-well plates (40,000 per well for A20 and HEK293 and 50,000 for HeLa) with 180  $\mu$ l medium per well. VHH7-DM1 fusion and free DM1 (10X, 20  $\mu$ l) were added to each well to get desired final concentrations (1  $\mu$ M, 0.5  $\mu$ M, 0.1  $\mu$ M, 0.05  $\mu$ M, 0.025  $\mu$ M, 0.015  $\mu$ M, 0.005  $\mu$ M). After incubation for 48 h at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>, A20 cells were suspended gently and transferred to a round bottom 96-well plate. The cells were spinned down and medium was removed followed by the resuspension in 100  $\mu$ L phenol red free medium. For adherent cells HEK293 and HeLa, old medium were aspirated directly followed by the addition of 100  $\mu$ L phenol red free medium. After changing to phenol red free medium. 10  $\mu$ L 12 mM MTT in PBS was added to each well and the plate was incubated at 37 °C for another 2 h. At the end of incubation, medium was removed and the cells were lysed by adding 50  $\mu$ L DMSO per well. The absorbance was read at 540 nm on a SpectraMax 190 plate reader (Molecular Devices).

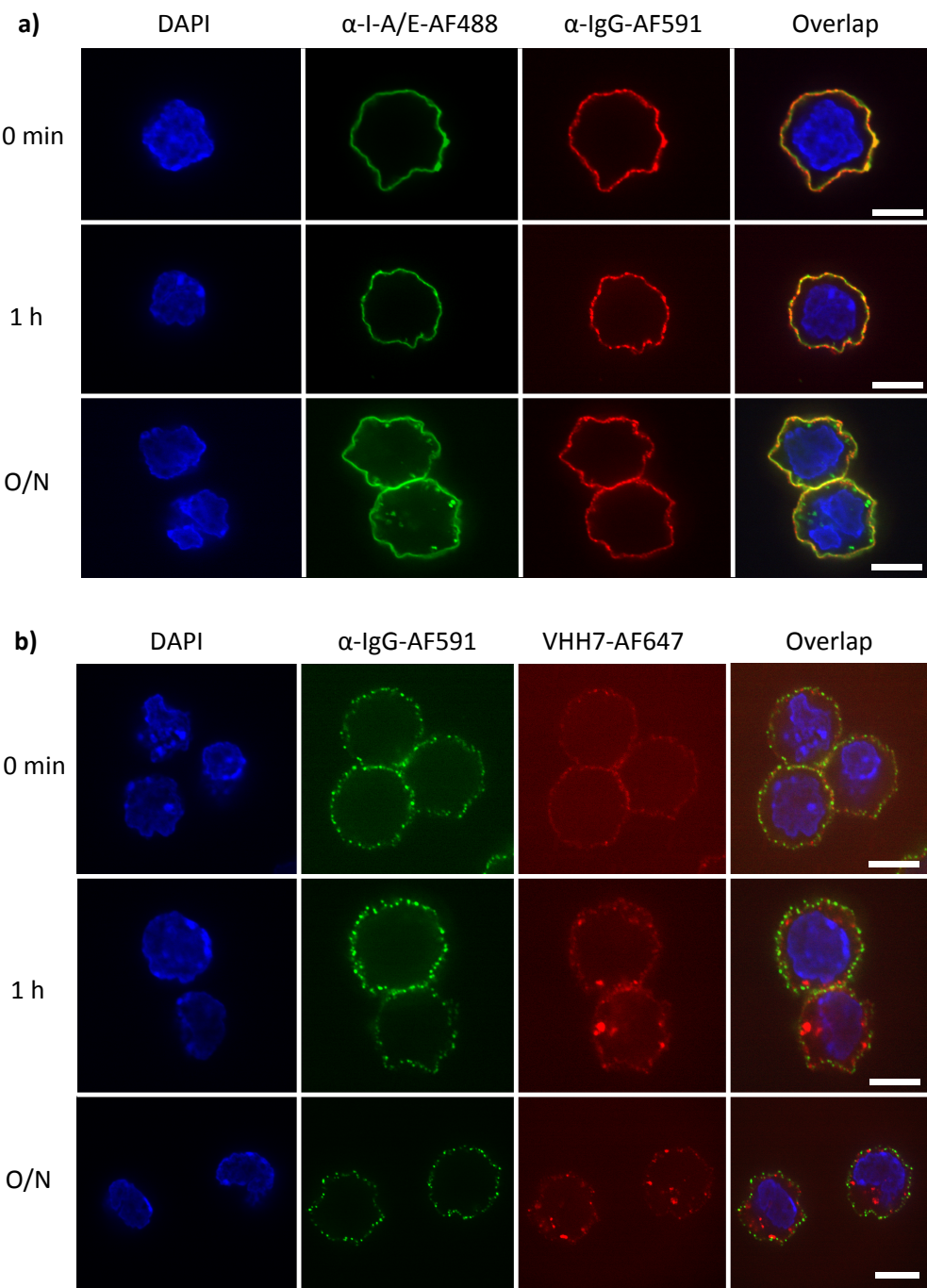


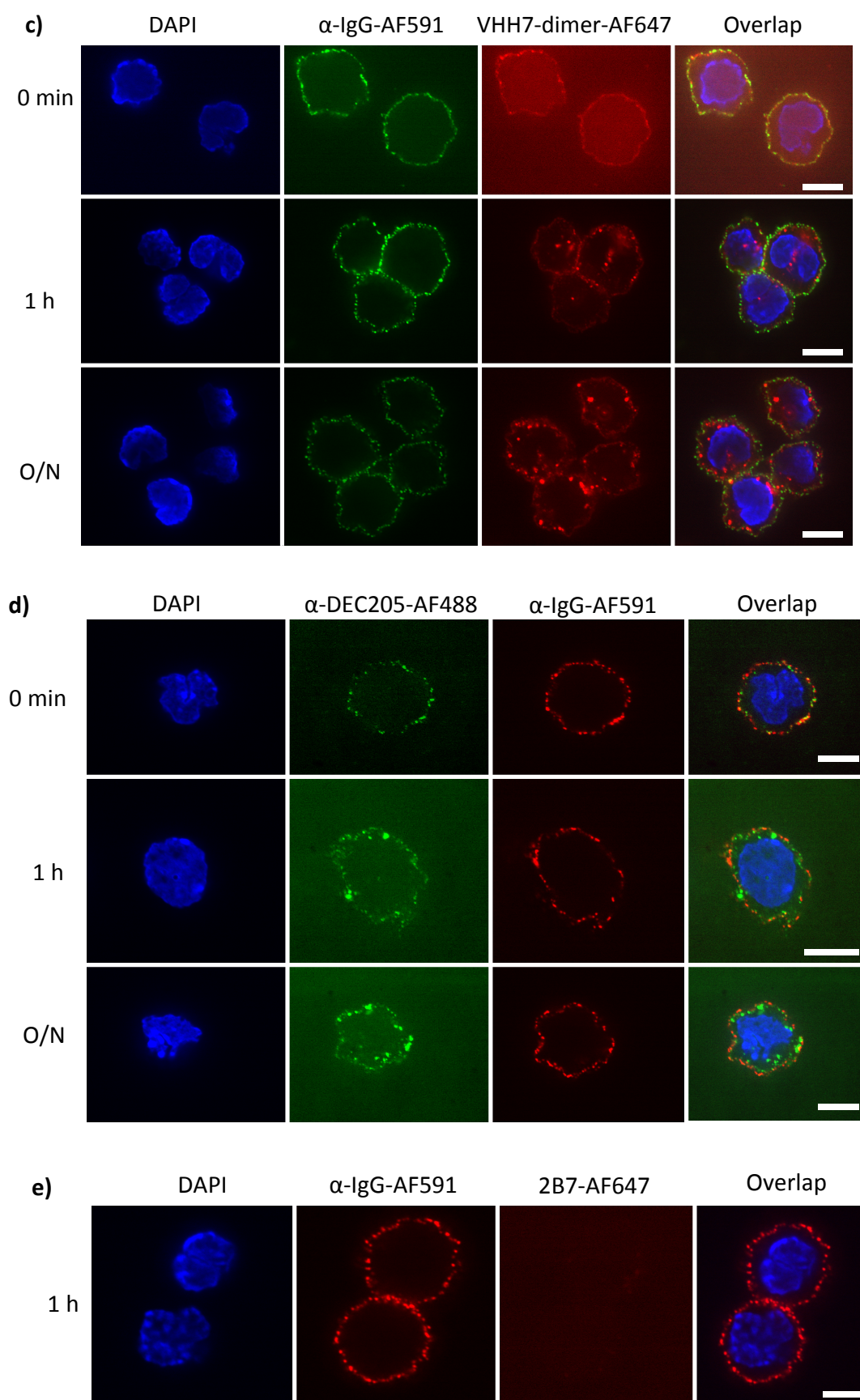
**Figure S2.** *In vitro* cytotoxicity MTT assay of VHH7-DM1 and DM1 on MHC-II positive (A20) and negative (HEK293, HeLa) cell lines. (Bars, means  $\pm$  SD, n=3, CI: confidence interval)

## 6. Time course studies of VHH7 internalization by confocal microscopy

8-well chamber slide (Lab-Tek II) was precoated with poly-L-lysine (Cultrex) and washed with PBS (3  $\times$  1 ml). A20 cells were seeded to each chamber by the addition of 300  $\mu$ l medium containing 25,000 cells. VHH7-AF647, commercial anti-mouse I-A/I-E AF-488 (clone: M5/114.15.2) and anti-mouse DEC205-AF488 (clone: 205yekta) were added to each chamber to final concentration of 50 nM at 16 h, 1 h, 10 min, 5 min, 0 min before the chamber was transferred from 37  $^{\circ}$ C incubator to ice surface. 500  $\mu$ l prechilled PBS was added quickly to each chamber, drained and repeated. For the surface staining, goat anti-mouse IgG(H+L) AF591 was added and incubate on ice for 20 min, then washed (PBS, 3  $\times$  1 ml). Cells were fixed by 4% paraformaldehyde in PBS (300  $\mu$ l) for 10 min on ice. After

washing, the upper chamber was removed followed by the addition of Vectashield mounting medium with DAPI before imaging on a Nikon spin-disk confocal. Images were analyzed on Velocity.





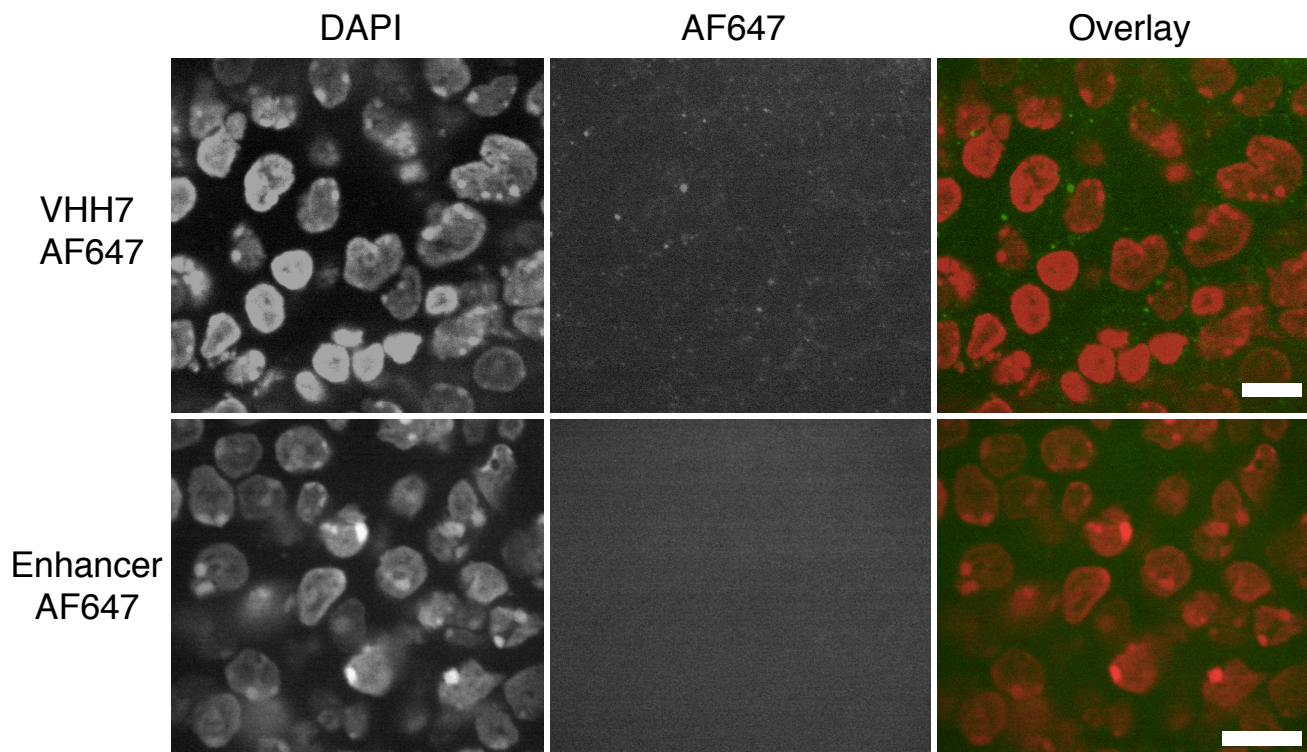
**Figure S3.** Rapid and epitope dependent internalization of VHH7 by murine A20 cells as revealed by confocal microscopy. ( $\alpha$ -IgG-AF591 served as membrane stain, bar = 10  $\mu$ m). a) Anti-mouse I-A/I-E



rabbit IgG2a  $\kappa$  (M5/114.15.2) is slowly internalized after overnight culture with limited vesicle structures. b) VHH7 is quickly internalized within 1 h. c) Genetic bi-valent VHH7 dimer is internalized at similar rate to monomeric VHH7 indicating the rapid internalization is epitope but not valent dependent. d) Anti-DEC205 (205yektA) as positive control for internalization. e) Irrelevant VHH 2B7 targeting human integrin as negative control.

## 7. Confocal imaging of tumor tissue sections

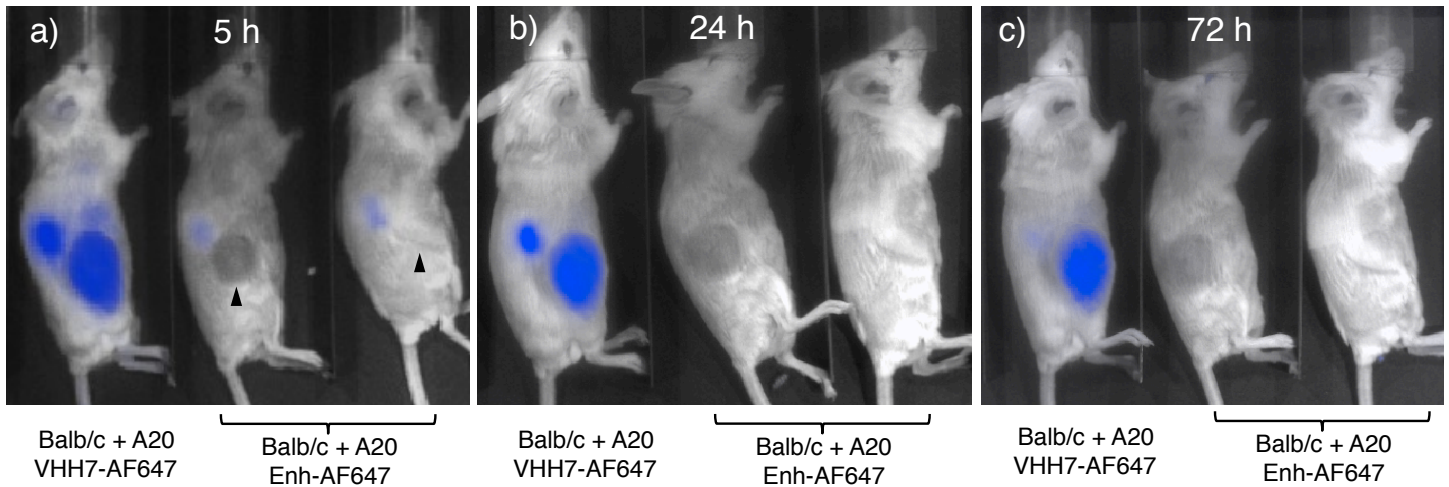
6 to 10 weeks old female Balb/c mice were inoculated with  $2.5 \times 10^6$  A20 cells subcutaneously. Once the longest dimensions of tumor burdens reach 10 mm (usually  $\sim 15$  days), VHH7-AF647 or Enhancer-AF647 conjugate (40  $\mu$ g) were injected through tail vein. After 2 h, the mice were sacrificed and the tumors were removed, emerged into a cryomold with O.C.T for 10 min before snap freezing. The tissue was cut into 30-micron slice on a Leica cryostat, covered with Vectashield mounting medium containing DAPI and directly imaged on a Nikon spin-disk confocal microscope. Images were analyzed on Velocity.



**Figure S4.** Tissue section of A20 tumor shows accumulation of VHH7-AF647, but not non-targeting Enh-AF647, in vesicle structures. (Images were collected and presented under identical conditions. Bar= 10  $\mu$ m)

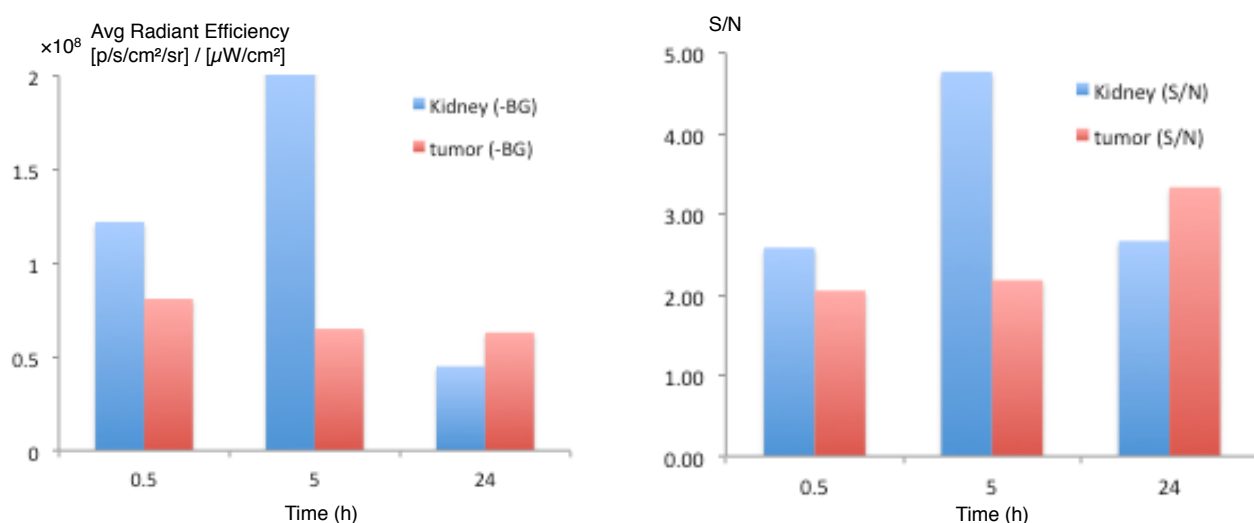
## 8. NIR imaging

Mice were inoculated with  $2.5 \times 10^6$  and  $1.5 \times 10^6$  A20 cells at the lower flank or through the tail vein respectively, and imaged until tumor burden reached 1 cm at the longest dimension or become palpable on liver in case of metastasized model (typically 3 weeks P.I.). Mice for imaging were maintained on Casein Diet 10 IF for at least one week. Before imaging, mice were shaved and maintained under continuous flow of isofluorane. Background images of mice were collected followed by the intravenous injection of 40  $\mu$ g VHH7/Enh-AF647. The images were collected on an IVIS spectrum optical imaging system (PerkinElmer) and analyzed by Living Image 4.4 software. General settings for collecting epi-fluorescent images were as follows: Epi-illumination,  $\lambda_{em}=680$ ,  $\lambda_{ex}=640$ , Bin: (M)8, FOV: 13.1, f-stop = 2, acquisition time = 1 s. In case of saturated pixels, either reduce acquisition time to 0.5 s or adjust the bin size to small. Images were collected at dorsal, ventral or side views.



**Figure S5.** Time course images of VHH7/Enh-AF647 injected A20 bearing mice. (color scale: full range, minimum to maximum). a) 5 h after initial injection, significant signal from tumor site and kidney were obtained from VHH7-AF647 injected mice. No nonspecific signals at tumor sites were observed from Enh-AF647 injected mice (black arrows: tumors without fluorescent signal). b) Complete clearance of Enh-AF647 was observed after 24 h for Enh-AF647 injected mice. The longer renal clearance of VHH7-AF647 may be due to the accumulation of detached VHH7 from tumor sites or other MHC-II<sup>+</sup> populations. c) Fluorescent signal from the tumor sites persisted after 72 h supporting the internalization of the VHH7-AF647 conjugate.





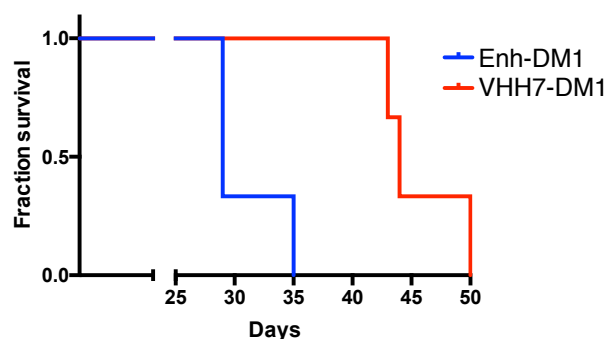
**Figure S6.** Pharmacokinetics of VHH7 conjugate *in vivo*. Mouse with subcutaneously inoculated A20 was injected with 40 ug VHH7-AF647 intravenously followed by IVIS imaging at different time points. (a) Average radiant efficiencies from size fixed regions of interest (ROI) were selected for tumor, kidney and a close area as background (BG); (b) The signal to noise (S/N) ratios were calculated based on the signal from ROI over BG.

## 9. Animal studies

BALB/cAnNCrI mice (Charles River Laboratories, Wilmington, MA) were injected with  $2.5 \times 10^6$  A20 cells in a total volume of 500  $\mu$ L HBSS. The cell preparations were given subcutaneously on the right flank of animals. Once established, the tumors were allowed to grow until the average volume of tumor reached a volume of 150 mm<sup>3</sup> at which point the animals were randomized and regrouped (3 animals per group) prior to treatment initiation. Tumor volumes were calculated using a modified ellipsoid formula:  $V = (\text{width})^2 \times \text{length} / 2$ . The native VHH7, VHH7-DM1 conjugate, or PBS were administered via tail vein injection. Tumor-bearing mice received a total of six doses at day 10, 12, 14, 16 and 20. Tumors were monitored everyday using calipers until mice were euthanized when the longest dimension reached 2 cm.

For disseminated lymphoma model, 6 Balb/c were inoculated with 1.5 million A20 cells through tail vein and randomized into two groups. Starts from Day 1, the two groups were injected with VHH7-DM1 or irrelevant Enh-DM1 conjugate respectively for a total of 4 injections every other day until Day 7. The mice were monitored daily and euthanized based on human endpoint when present one of the

following criteria 1) inability to remain upright; 2) prolonged decreased food intake; 3) enlarged lymph nodes. Survival rate was shown in Figure S7.

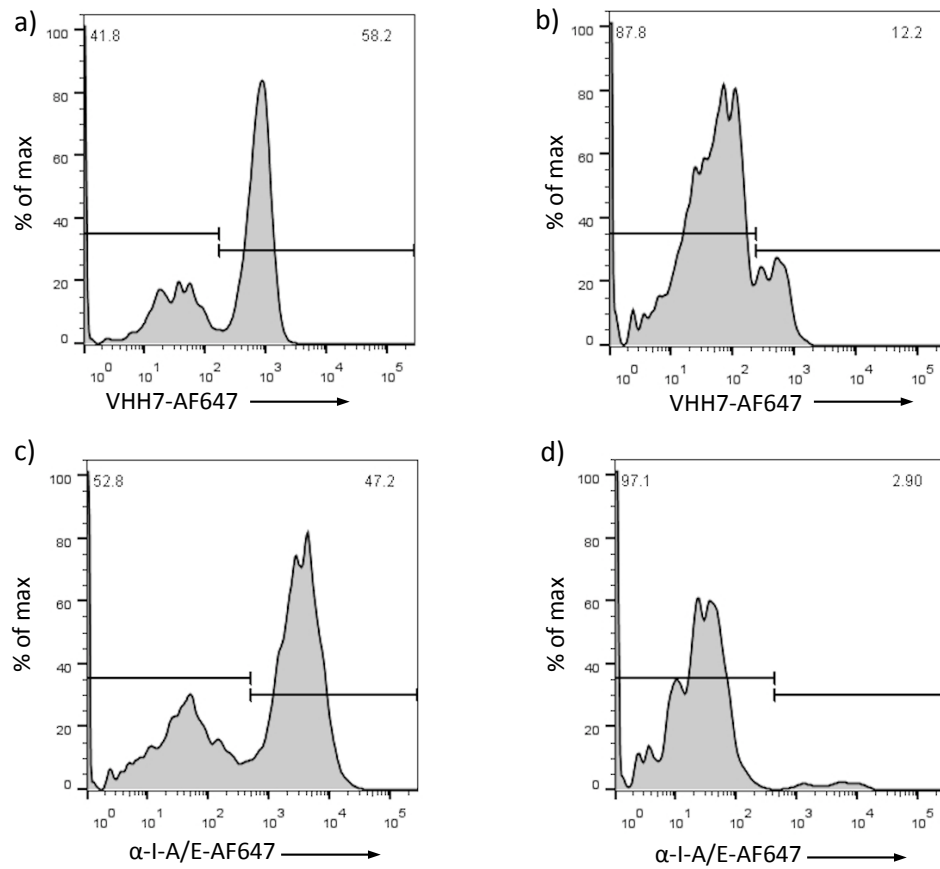


**Figure S7.** Percentage survival of Balb/c bearing disseminated A20 lymphoma. Median survivals: 29 days (Enh-DM1), 44 days (VHH7-DM1). (n=3)

## 10. Comparison of biodistribution of VHH7 with full-sized $\alpha$ -I-A/E antibody

For injection at the same fluorescence dose (RFU 6400) (Figure 3h), AF647 labeled full-sized antibody and VHH7 were added to a microtiter plate to the same final volume (100  $\mu$ l). In this case, full-sized antibody gave stronger fluorescence intensity; therefore the amount of VHH7 injected was adjusted proportionally. 4 h after i.v. injection, the tumors were dissected, incubated with collagenase D, and passed through 45  $\mu$ m cell strainer to make a single cell suspension prior to FACS staining and analysis.

For injection at the same molar dose (0.3 nmol), VHH7 and full-sized antibody were injected intravenously. Inguinal lymph nodes and spleens were dissected 2 h later, incubated with collagenase D, and passed through 45  $\mu$ m cell strainer to make a single cell suspension prior to FACS staining and analysis.



**Figure S8.** FACS analysis of VHH7 and full-sized antibody injected at equal molar dose. a) spleen staining by VHH7; b) inguinal lymph nodes staining by VHH7; c) spleen staining by  $\alpha$ -I-A/E; d) inguinal lymph nodes staining by  $\alpha$ -I-A/E.